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(54) Title: METHOD FOR IMMOBILIZING HAPTENS ON A TEST ARTICLE

(57) Abstract

A method for preparing test articles comprises immobilizing a hapten-carrier molecule conjugate on a solid phase surface. Attachment of a hapten is effected through the carrier molecule which non-covalently adsorbs to the surface. The hapten is a small molecule which retains binding activity even under relatively harsh conditions which would degrade more labile substances such as antibodies and other binding proteins. Such labile binding proteins may subsequently be introduced to the solid phase through binding with anti-hapten binding substance under mild conditions. Optionally, the carrier molecule can be covalently attached to the surface after initial non-covalent position, for example by radiation-induced cross-linking.

METHOD FOR IMMOBILIZING HAPTENS ON A TEST ARTICLE

BACKGROUND OF THE INVENTION

1. Field of the Invention:

The present invention relates generally to the production of test articles used for determining the presence of analyte in biological samples. More particularly, the present invention relates to a method for immobilizing haptens onto solid phase surfaces.

A wide variety of assay techniques and methods exist for determining the amount of a target analyte in a biological or other sample. Specific binding assays rely on detecting an analyte using a specific binding substance which reacts with the analyte in a highly selective manner. Numerous protocols and formats for performing such specific binding assays are described in the patent, technical, and medical literature.

Of particular interest to the present invention, many specific binding assays employ a solid phase surface having a specific binding substance, such an antibody or antibody fragment, immobilized on the surface. The surface may then be utilized to capture analyte through direct or indirect binding of the immobilized specific binding substance to the target analyte.

Immobilization of the specific binding substance on the solid phase surface can be problematic in a number of respects. First, the specific binding substance must be applied and attached to the surface under conditions which do not result in loss of binding activity, e.g. conditions which do not result in denaturing of antibodies or other binding protein. Thus, a variety of otherwise desirable application techniques, such as ink jet printing, are difficult to use since they can denature labile proteins, such as antibodies. Second, many antibody binding protocols which are sufficiently gentle to avoid denaturing the binding substances require

multiple steps which are time consuming and labor intensive,
making preparation of the solid phase surface expensive. The
expense is further increased when it is desired to form a
plurality of discrete, frequently very small, reaction zones
5   on a single solid phase surface. In addition, many specific
binding substance immobilization techniques require
considerable time to achieve maximum binding of the substance
to the solid phase surface. Attempts to shorten the binding
time often result in the desorption or sloughing of the
10 substances from the test surface.

For these reasons, it would be desirable to provide
improved methods for immobilizing specific binding substances
on solid phase surfaces to produce test articles useful for
performing biological and other assays. In particular, it
15 would be desirable to provide improved fabrication methods
where the specific binding substance can be attached in a
rapid manner over very small areas on a surface using
accurately and precisely measured volumes of a binding
substance to define a reaction zone. It would be further
20 useful to provide methods for immobilizing binding substances
which can be performed using conventional printing methods and
equipment, such as ink jet printers of a type which can
subject the material being deposited to relatively harsh and
often denaturing conditions. The methods of the present
25 invention are preferably able to utilize materials which can
withstand such denaturing conditions, allowing the preparation
of test articles having multiple and precisely defined
reaction zone patterns which can be achieved using printing
techniques. The ability to employ conventional printing
30 techniques provides for economic fabrication of test articles,
even when the test articles require the complex and precise
formation of reaction zones on the solid phase test surface.

2. Description of the Background Art

The printing of chemical and biological reagents on
35 surfaces is described in a number of patent publications,
including U.S. 4,046,513 (reagents); 4,366,243 (haptens);
4,877,745 (ink jet printing of biological reagents such as

allergens); 5,108,926 (ink jet printing of viable cells);
5,260,195 (ink jet printing of a polymer binder and enzyme
coating mass); 5,326,691 (ink jet printing of cells and cell-
binding agents); and JP6/041483 (Abstract) (printing inks for
5   jet printers which incorporate proteins to modify appearance).
Photolithographic fabrication of biosensor surfaces is
described in U.S. Patent Nos. 5,212,050; 5,200,051; and
5,063,081. U.S. Patent No. 5,316,784, describes a two-step
process for attaching immunologically active substances, such
10   as antibodies, antigens, and binding proteins, to solid phase
surfaces by applying a mixture of the active substance and a
linking group having photoactivable groups to the surface.
After allowing the mixture to absorb into the surface, the
active substance is covalently attached by activating the
15   linking group. U.S. Patent No. 5,258,041, describes the use
of spacer arms having hydrophobic guiding groups for attaching
biomolecules to solid phase supports.

SUMMARY OF THE INVENTION

Methods for preparing solid phase surfaces for use
20   as test articles in biological and other assays comprise
immobilizing a hapten on the solid phase surface via a carrier
molecule, typically a macromolecule such as a protein, which
is capable of non-specific adherence to the surface. A
conjugate, of the hapten and carrier molecule typically
25   covalently attached, is applied to the surface so that the
carrier molecule adheres to and the hapten is immobilized
precisely at the site of application. The hapten is a small
molecule which will retain binding activity even under
relatively harsh conditions of application that would denature
30   antibodies and other specific binding proteins. While the
carrier molecule, which may be a protein, might be denatured
under such conditions, loss of biological activity of the
carrier molecule is not significant since it will still
provide for the necessary non-specific adherence to the solid
35   phase surface and preservation of the attached hapten.

In some cases, the test articles with immobilized
hapten may find use in assays where the hapten is used to bind

directly or indirectly to an analyte of interest, typically
where the analyte has previously been bound to anti-hapten
antibody. Alternatively, the test article may be further
prepared by introducing a specific binding substance, usually

5   an antibody or antibody fragment, to the hapten via an anti-
hapten linker moiety. Preferably, a covalently attached
conjugate of the specific binding substance and the anti-
hapten binding substance may be reacted with the solid phase
surface of the test article under relatively mild (non-

10   denaturing) conditions to form a non-covalent linkage between
the hapten and the specific binding substance.

Such fabrication methods are preferably performed
using printing processes where discrete droplets of a liquid
medium containing the hapten-carrier molecule conjugate are

15   applied to the solid phase surface to form precise and
accurately defined reaction zones, often in complex geometric
patterns which would be difficult to produce using other
techniques. The printing processes also allow for application
of different haptens within different reaction zones, to

20   produce test articles which are useful in multiple analyte
assays. In a preferred aspect of the present invention,
particularly ink jet printing is employed where the liquid
medium is contained in a reservoir and the droplets are
propelled or ejected through an aperture by known ink jet

25   printing technologies. Both piezoelectric and thermal ink jet
printing systems may be used for performing the methods of the
present invention.

Test articles according to the present invention
will have a solid phase test surface having hapten immobilized

30   thereon via a carrier molecule. The carrier molecule will
usually be non-specifically attached to the solid phase test
surface, but in some cases, it may be desirable to further
covalently cross-link the carrier molecule to the surface
(using conventional cross-linking methods employed after the

35   initial non-covalent attachment). Such further covalent
attachment has several advantages. First, cross-linking of
the carrier can reduce the time necessary to achieve high
affinity or avidity binding of the complex to the solid phase.

Second, presence of the carrier molecule reduces the risk of
denaturing the hapten which provides the active binding site
in the complex. Third, the chance of desorption or other loss
_of the complex is greatly reduced. The solid phase may be
5   composed of any material conventionally used in assay systems,
such as organic polymers, glasses, ceramics, metals,
absorptive papers, and the like. The haptens and the carrier
molecules will generally be as described above.

BRIEF DESCRIPTION OF THE DRAWINGS

10   Fig. 1 is a schematic illustration of an ink jet
printing system adapted for the printing of hapten-carrier
molecule conjugates on a solid phase disk surface according to
the method of the present invention;

           Fig. 2 is an electrical schematic diagram showing
15   the components of the head drive electronics of the ink jet
printing system of Fig. 1;

           Fig. 3 is an illustration of a biotin-BSA printed
disk showing discrete patterns and demonstrating the retention
of streptavidin binding activity;

20   Fig. 4 is a graph illustrating the desorption of
biotin-BSA as a function of liquid-phase adsorption time;

           Fig. 5 is a graph illustrating the amount of time
required for maximal biotin-BSA binding to the acrylic test
surface once spotted with the ink jet system;

25   Fig. 6 is a graph illustrating the acceleration of
biotin-BSA binding by heat treatment; and

           Fig. 7 is a graph illustrating the results of an
assay for CKMB using a solid phase test article prepared
according to the present invention, wherein binding of CKMB to
30   the test article is determined both interferometrically and
via light absorption.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

           Methods are provided for attaching haptens to solid
phase surfaces to form test articles useful for detecting
35   target analytes in biological and other assays. The target
analyte will be a member of a specific binding pair (SBP),

including compounds, compositions, aggregations, and virtually
any other substance which may be detected or reacted by
immunological or equivalent techniques. That is, the analyte,
or a portion thereof, will usually be antigenic or haptenic,
defining at least one epitopic site, or will be a member of a
naturally-occurring binding pair (e.g., carbohydrate and
lectin, hormone and hormone receptor, ligand and anti-ligand,
and the like). Analytes of particular interest include
antigens, antibodies, proteins, glycoproteins, carbohydrates,
macromolecules, toxins, bacteria, tumor markers, and the like,
which define a plurality of epitopic sites. Other analytes of
interest include haptens, drugs, and other small molecules,
which usually define only a single epitopic binding site. A
non-exhaustive list of exemplary analytes is set forth in U.S.
Patent No. 4,366,241, at column 19, line 7, through column 6,
line 42, the disclosure of which is incorporated herein by
reference. Of particular interest to the present invention is
the detection of cardiac markers, such as troponin I,
troponin T, myoglobin, and creatine kinase isozymes.

        The analytes may be present in a wide variety of
samples, where the sample is liquid, can be liquified, or can
be suspended in a liquid. The methods of the present
invention will find their greatest use with biological
specimens such as blood, serum, plasma, urine, cerebral fluid,
spinal fluid, ocular lens liquid (tears), saliva, sputum,
semen, cervical mucus, scrapings, swab samples, and the like,
which are frequently employed in the diagnosis and monitoring
of disease and therapeutic treatments. In addition, the
methods of the present invention may be used with industrial,
environmental, and food samples, such as water, process
streams, milk, meat, poultry, fish, conditioned media, and the
like. In certain circumstances, it may be desirable to
pretreat the sample, such as by liquification, separation,
solubilization, concentration, filtration, chemical treatment,
or a combination of these steps, in order to improve the
compatibility of the sample with the remaining steps of the
assay, as described hereinafter. The selection and
pretreatment of biological, industrial, and environmental

samples prior to immunological testing is well known in the
art and need not be further described.

The haptens which are to be immobilized on the solid
phase surface are small molecules, typically being below

5   2000 Daltons, usually being below 1000 Daltons, frequently
having a molecular weight in the range from 100 to
500 Daltons, and more frequently having a molecular weight in
the range from 200-500 Daltons. Such small molecules will
generally be resistant to the denaturing conditions which may

10   be present in the methods of the present invention,
particularly when ink jet printing mechanisms are employed,
and will thus remain stable and biologically active so that
they can bind their specific binding partner. While ink jet
printing mechanisms differ significantly in their designs,

15   such mechanisms can subject the material to be deposited to a
variety of conditions which can denature labile materials,
such as localized heating (including boiling of the liquid
medium carrying the material to be deposited), substantial
shear forces which can physically disrupt larger molecules, as

20   well as adverse drying conditions utilized after the material
has been deposited.

The haptens should possess a high-affinity binding
partner, preferably having a binding affinity of at least
about 10^{-8} M^{-1}, more preferably being at least 10^{-10} M^{-1}, and

25   most preferably being 10^{-11} M^{-1} or higher. The haptens should
be suitable for attachment, usually covalent attachment, to
the carrier molecule, as described in more detail hereinbelow.
Exemplary haptens include biotin (which binds to avidin,
streptavidin and anti-biotin antibody with very high

30   affinity), fluorescein (which binds with high affinity to
anti-fluorescein antibody), dinitrophenol (which binds with
very high affinity to anti-dinitrophenol antibody), digoxin
(which binds with very high affinity to anti-digoxin
antibody), luminol (which binds with very high affinity to

35   anti-luminol antibody), theophylline (which binds with very
affinity to anti-theophylline antibody), morphine (which binds
with very high affinity to anti-morphine antibody), and the
like.

Carrier molecules useful in the methods of the present invention will typically be macromolecules having a molecular weight of at least about 10 kiloDaltons (kD) with no upper size limit, usually being at least about 20 kD, and

5   typically being in the range from about 20 kD to 200 kD. Particularly suitable are large proteins which have high non-specific binding affinities for the solid phase surface of interest. Such proteins, however, should not contribute to the non-specific binding of other substances to the surface

10   after they have been immobilized thereon. Exemplary carrier proteins include albumins, ovalbumins, immunoglobulins, thyroglobulins, ferritin, and the like. Particularly preferred carrier proteins include serum albumins, such as bovine serum albumin (BSA), human serum albumin (HSA), and the

15   like.

The hapten and carrier molecule will be conjugated to each other to form a hapten-carrier molecule complex prior to application to the solid phase surface. Such conjugation will usually be achieved through covalent binding using

20   conventional chemistries depending on the natures of both the hapten and the carrier molecule. Frequently, covalent binding will be achieved using a bivalent cross-linking substance which is capable of attaching at one end to the hapten and at the other end to the carrier molecule. Such substances

25   include succinimides, carbodiimides, glutaraldehydes, diazotization coupling, and the like. These and other suitable coupling chemistries are well-described in the biological and chemical literature. See, for example, Avrameus et al. (1978) Scand. J. Immunol. 8:7-23. The

30   resulting conjugates will typically have hapten numbers of at least 1, more usually of at least 2, preferably being at least 4, often being in the range from 4 to 50, and preferably being in the range from 6 to 20.

The hapten-carrier molecule conjugates will be

35   present in a liquid medium suitable for direct application to the solid phase surface of the test article. The liquid medium will typically be aqueous, more typically being buffered within a desired pH range, usually from pH 5 to pH 9.

Phosphate-buffered saline (PBS) is a suitable liquid medium
for most hapten-carrier molecule conjugates.

　　　The liquid medium may contain other components for
achieving desired purposes. For example, liquid media may
5   include preservatives, such as sugars, intended to enhance
stability of the conjugate during application. Surprisingly,
however, it has been found that the hapten conjugates of the
present invention generally do not require preservative or
other components to maintain stability of the haptens in the
10   printing methods of the present invention. The ability to
dispense with preservatives (which can interfere with binding
of the carrier molecule to the surface) is a particular
advantage of the present invention.

　　　The liquid medium will typically have a hapten-
15   carrier molecule concentration in the range from about 1 μg/ml
to 250 μg/ml, preferably from 5 μg/ml to 50 μg/ml, with
viscosities equivalent to water, preferably being about 1 cp
to 2 cp. It is possible to add viscosity-enhancing
components, such as glycols, polyvinyl alcohols, or the like,
20   where the viscosity is increased to about 5 to 10 cp,
resulting in improved fluid dispersion characteristics from an
ink jet printing head. The use of viscosity-enhancing
reagents, however, can interfere with protein adsorption
particularly to plastics where it will interfere with non-
25   specific hydrophobic interactions between a protein and a
plastic surface. Thus in many cases, the addition of such
viscosity-enhancing components can decrease the amount of
active hapten-carrier molecule adsorbed onto the solid phase
surface, and the addition of viscosity enhancing components is
30   not generally preferred at present.

　　　A preselected volume of the liquid medium (typically
from 200 pl to 100 μl) containing the hapten-carrier molecule
conjugates may be applied to the solid phase surface in any
manner that results in an initial non-covalent attachment of
35   the carrier molecule to the surface. For example, the liquid
medium may be applied by pipetting, including micropipetting
(volumes in the range from about 200 pl to 100 μl), spraying,
printing, dipcoating, spin coating, stamping, or the like.

The preferred and exemplary method for applying the liquid
media comprises printing using a conventional or modified ink
jet printing mechanism where the liquid medium is initially
contained in a reservoir and dispensed therefrom in discrete

5   liquid droplets (typically each having a volume in the range
from 10 pl to 1 μl) using piezoelectric, thermal, or other
dispensing mechanisms.

         Ink jet printing technology is well known in the art
and described in numerous patents. See, for example, U.S.

10   Patent Nos. 4,240,081; 4,475,113; 4,533,082; 4,544,933;
4,591,883; and 5,164,740, the full disclosures of which are
incorporated herein by reference. Typically, the fluid to be
printed (which is the liquid medium in the case of the present
invention) is ejected through a small aperture under pressure

15   and transformed into uniform droplets by one of various known
ink jet printing mechanisms. A preferred class of ink jet
printer relies on an evaporative volume increase produced by
thermal cycling of the liquid medium. Such ink jet printing
mechanisms are available commercially from Hewlett-Packard,

20   Palo Alto, California. Other ink jet printing mechanisms
which may find use rely on vibration of a piezoelectric
crystal or thermal effect to produce the droplets, where
droplets may be charged electrostatically and deflected by a
control system, typically a computer. Using either system,

25   the resulting pattern of applied liquid medium can be
precisely and accurately controlled. As described in detail
in the Experimental section hereinafter, the ink jet printing
mechanism will typically be combined with a mechanism for
supporting the solid phase surface to be derivatized. In this

30   way, the solid phase can be automatically relocated relative
to the ink jet printing head in order to print the liquid
medium (which contains the hapten-carrier molecule conjugate)
to different locations on the solid phase surface. Thus, a
plurality of different reaction zones can be formed on the

35   solid phase surface. Moreover, by employing multiple
reservoirs for containing different hapten-carrier conjugates,
or alternatively by refilling a single reservoir with
different hapten-carrier molecule conjugate(s), reaction zones

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having a variety of different activities can be formed on a
single solid phase surface.

The solid phase surface employed in the methods of
the present invention will include at least a single reaction
5   zone, often including two or more reaction zones, frequently
including from 3 to 100 reaction zones, and often including
from 4 to 50 reaction zones. The reaction zones may be
specific for a single analyte, or different reaction zones may
be specific for different analytes. It will be appreciated,
10   of course, that the reaction zones may be capable of directly
or indirectly, competitively or non-competitively binding, or
providing any other type of binding compatible with the assay
formats of the present invention.

The reaction zones are usually optically flat to
15   facilitate spectrophometric, interferometric, fluormetric or
other interrogation thereof. The reaction zones will usually
have a minimum area of 0.01 mm^2, usually being from 0.1 mm^2 to
100 mm^2, and preferably from 0.1 mm^2 to 5 mm^2. For example, a
plurality of circular reaction zones having areas in the range
20   from 0.1 mm^2 to 5 mm^2 may be formed on a single flat surface
of a disk substrate. When the reaction zone(s) are formed by
the application of discrete droplets of the liquid medium, the
total number of droplets will usually be in the range from 1
to 10,000, preferably being from 500 to 2,500, for an
25   approximately 1 mm diameter spot.

The substrates which provide the solid phase surface
may be composed of a variety of conventional materials,
including organic polymers, glass, ceramics, metals, and the
like. For use with interferometers, substrates will
30   preferably be transparent or translucent, and will be composed
of organic polymers. An exemplary material is
methylmethacrylate polymer.

After applying the liquid medium containing the
hapten-carrier molecule conjugate to the solid phase surface,
35   either by ink jet printing or other techniques, the surface
may be treated to enhance adherence of the carrier molecule to
the surface. It has been found that the solid phase surface
may be at a temperature above room temperature, typically at

least 50°C, and often 90°C, or higher, to provide
substantially accelerated and enhanced adherence of the
carrier molecule to the surface. Such high temperature
treatment is typically performed for a relative short period
5   sufficient to maximize binding of the complex to the surface,
usually from 10 minutes to 1 hour. While such conditions
would inactivate many antibodies and other specific binding
materials, the hapten-carrier molecule complexes of the
present invention will remain active and able to bind their
10   binding partners even after exposure to heat and other
potentially denaturing conditions.

In addition to, or as an alternative to, heating the
surface to enhance non-covalent bonding, it is also possible
to covalently cross-link the carrier molecules to the solid
15   phase surface by subjecting the adsorbed surface to suitable
conditions, e.g., light, radiation, heat, cross-linking
reagents, or the like. It should be noted that such covalent
cross-linking of the molecule occurs after initial
immobilization (usually non-covalent attachment) has been
20   effected. A particular advantage of the present invention is
that the hapten may be accurately and precisely adhered to the
solid phase surface without the need to previously derivatize
the surface for covalent linkage. Under certain
circumstances, however, it may be desirable to provide for
25   such covalent linkage after the reaction zones have been
initially defined non-covalently.

In a preferred aspect of the present invention, the
hapten-carrier molecule will be further derivitized to include
a cross-linking moiety, preferably a photo-activatable group
30   such as a benzophenone of fluorinated aryl azide. After
adsorption of the complex to the solid phase, usually a
plastic surface, ultraviolet radiation can be directed at the
solid phase surface to convert the cross-linking groups to
reactive intermediates which will insert into the carbon-
35   hydrogen bonds of the plastic surface. In order to accelerate
the cross-linking, the complexes will be highly substituted
with the reactive groups on the carrier molecule (typically
from 1 to 25, preferably from 5 to 15 substitutions per

molecule) and high doses of the ultraviolet radiation can be
applied to the surface (typically from 100 to 1,000 $\mu W/cm^2$ at
6 inches from the surface). While denaturation of
biologically active materials is likely under these conditions

5   (because of formation of multiple linkages and the random
distribution of the covalent bonds to the surface, as well as
the susceptibility of many molecules to ultraviolet light),
the hapten-carrier molecule complex of the present invention
has been found to be significantly less susceptible to such

10   denaturation. The formation of multiple links between the
carrier molecule and the plastic surface will not
significantly reduce the binding activity of the hapten.
Moreover, the hapten and carrier molecule may be selected for
optimal ultraviolet light tolerance, preferably both having

15   low absorption in the UV range around 280nm.
          After the hapten has been immobilized on the solid
phase surface via the carrier molecule, the resulting test
article may be used directly in certain assay formats. For
example, assays which rely on introducing an anti-hapten

20   binding substance to a target analyte using antibody or other
binding substance specific for the target analyte can utilize
the test article having the immobilized hapten as a capture
device. For example, avidin bound to a target-analyte
specific antibody may be reacted with the test sample. The

25   resulting complex of analyte, antibody and avidin may then be
captured using a test article having biotin hapten immobilized
on its test surface. After separating the solid phase from
the test sample, the presence of the target analyte can be
confirmed using antibody attached to a label in a conventional

30   "sandwich" assay format.
          The test articles of the present invention may
alternatively be further derivatized to have a specific
binding substance of interest immobilized to the test surface.
Such immobilization may be readily accomplished by reacting

35   the solid phase surface under mild (non-denaturing) conditions
with a solution containing a specific binding substance, such
as an antibody or antibody fragment, covalently attached to
the anti-hapten binding substance. In this way, the specific

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binding substance is precisely and accurately bound to the
reaction zone(s) defined by the immobilized hapten under mild
conditions which do not result in loss of activity of the
specific binding substances. Such test articles are useful in
5   a variety of conventional assay formats which employ direct
binding between the immobilized specific binding substance and
a target analyte.

The following examples are offered by way of
illustration, not by way of limitation.

10   **Experimental**

1.   Immobilizing Biotinylated BSA to Acrylic Test Articles
A.   Jetting System Description

A system for ink jet printing of haptenated
macromolecules is shown in Figs. 1 and 2. The system includes
15   a HP Model 51616A Ink Jet Head 10 (Hewlett Packard Ink Jet
Components Division, Corvallis, OR). To facilitate injecting
haptenated solutions into the jetting chambers 12 of the head
10 and to avoid contamination of the solutions, the face plate
of Model 51616A was obtained sans the molded part which
20   normally forms the ink reservoir. A fluid tube 14 was then
easily connected between a 15 mL polypropylene test tube 16
containing the haptenated solution and the entrance to the
jetting chamber 12. The head 10 was mechanically and
electrically interfaced using a HP Model 51610A Carriage
25   Assembly 18 (Hewlett Packard Ink Jet Components Division,
Corvallis, OR). The resulting assembly was mounted on X-axis
and Y-axis micrometer slides 20 and 22 to facilitate
positioning the head precisely over an acrylic disc D on which
the solutions were to be printed. Prior to printing, the
30   acrylic disc D was installed on a round disc mounting platen
24 via insertion on a center locating pin. The disc mounting
platen 24 was attached to a motor shaft of stepper motor 26
(Parker-Compumotor, Harrison City, PA, Model # S57-51-MO),
allowing the disc to be rotated under computer control. The
35   stepper motor 26 was mounted on a Z-axis slide 30 which
allowed a stepper motor 28 (Parker-Compumotor Model # S57-51-
MO) to drive the platen 24 in the Z direction. This

facilitated lowering the disc D for removal and allowed the
vertical distance between the head and the disc, a critical
parameter in reproducible printing, to be replicated run to
run.   The number of droplets ejected from the head 10 to form
5   each deposited haptenated macromolecule spot, as well as the
energy pulses which caused the thermal head to fire were
controlled by head drive electronics 31 (see Fig. 2). Stepper
motors were controlled using two S-6 Series Microstep stepper
motor controllers 32 (Parker-Daedal, Harrison City, PA). The
10   stepper motor controllers 32 were interfaced to computer 34
using an AT-MIO-16X DAQ acquisition board 36 (National
Instruments Corporation, Austin, Texas). Control software was
written to control the motor motions using a LabVIEW software
package (National Instruments Corporation, Austin, Texas).
15   Fig. 2 shows details of the head drive electronics
31 and of the electrical schematic of the HPO Model 51616A Ink
Jet Head 10. The purpose of the head drive electronics is to
(1) supply a controlled impulse of energy to a selected
thermal resistor 40 inside the ink jet head, (2) control the
20   rate at which pulses are input to the head, and (3) control
the number of energy pulses delivered to the head in a
sequence thus controlling the amount of fluid deposited in one
spot on the acrylic disc D. The circuit functions as follows.
The ink jet printing technology relies upon heating the fluid
25   contained in small chambers inside the ink jet head. This
heating is accomplished by passing electrical current through
small resistors 40 embedded in the fluid chambers. As shown
in the schematic, the HP Model 51616A Ink Jet Head 10 has
thermal heating resistor values of approximately 65 ohms.
30   Each of the 12 orifices in this head has a heating resistor 40
localized above it. The 12 resistors are connected internal
to the head in the network shown in Fig. 2. Internal wiring
resistances result in the parasitic resistances also shown in
Fig. 2. All chamber heating resistors are connected to one
35   common line. To drive the head, MOSFET transistor 42 (BUZ71;
Siliconix, Santa Clara, CA) is turned on under control from
NPN transistor 44 (2N3904; Motorola, Phoenix, AZ) when a high
logic state is output from monostable multivibrator 46

(74LS123; Texas Instruments, Dallas, Texas). The monostable
multivibrator 46 controls the time that MOSFET transistor 42
is allowed to stay on allowing electrical current to pass
through the chamber heating resistor 40 in the ink jet head
5   10.

Resistor 48 (20 kΩ) is adjusted to control the pulse
period and tune the head for optimal jetting of the solution
of interest. A pulse width between 3 and 3.5 microseconds was
found to be optimal for jetting the solutions of interest.
10   The common node of the ink jet head is supplied with a 23 volt
DC potential from power supply 50 (Model LPS152, Leader
Instruments, Hauppauge, NY). This voltage is held constant
under all operating conditions using the monostable
multivibrator 46 to control the energy input to the chamber
15   heating resistors 40. The number of pulses of energy supplied
to the head 10 to form one spot is controlled by 12 bit pre-
loadable binary counter 52 which was loaded with the number of
droplets to be printed using 12 SPST switches 54. A 2 KHz
clock source 56 is input to the counter 52 and is used to
20   count down to zero from the pre-loaded number. The resulting
output occurs at a 1 KHz rate, half the input clock rate. The
maximum rate at which the HP 51616A ink jet head 10 has been
specified to be driven is 1.25 Khz, though it can be operated
up to 3 to 4 Khz for accelerated deposition rates. Even
25   higher jetting rates cause inconsistent jetting and damage to
the head (resistor failure). The line connected from the
counter 52 to the computer interface 36 is used to command the
head drive electronics 31 to deposit a spot on the acrylic
disc D after the stepper motors 26 and 28 have positioned the
30   disc D in the appropriate position.

B.   Acrylic Disk Preparation
35 mm acrylic disks used for substrate material were
obtained from the Germanow-Simon company. They were machined
from Hesalite HTC material and were 1.1 or 1.4 mm thick with a
35   high-tolerance 2.4 mm center hole. This material has optical
properties that are sufficient for interferometric and/or
fluorescent interrogation and protein binding characteristics

enabling immunoassay development on it. Disks were shipped
from the vendor laminated on both sides to minimize scratching
during machining, transport, and storage. Prior to ink jet
spotting, laminates were removed, and the disks were
5   (occasionally washed with mild detergents and) rinsed with
deionized water both to quench static electricity build-up and
to remove debris remaining from the machining process.

C.   **Preparation of Biotinylated BSA solutions**
Bovine serum albumin (BSA) was covalently attached
10   to biotin moieties (biotinylated) starting with 60 mg of a
10 mg/mL solution of BSA in phosphate buffered saline (PBS,
10 mM, pH 7.4,). The BSA was then conjugated at a molar ratio
of ca. 10:1 NHS-LC-biotin to BSA. For 60 mg of BSA, 4.1 mg of
NHS-LC-Biotin (Pierce #94052374) was dissolved in 60 μL of DMF
15   (Pierce #931026155) and added to the 6 mL of BSA in PBS. This
reaction mixture was placed in an ice bath for 2 hours
followed by placing the mixture in a 12,000 MWCO dialysis
tubing and dialyzing against 0.1 M phosphate buffer (pH 7.0,
with at least two 1 L changes) at 4°C to remove unreacted
20   biotin. Following dialysis and hapten number assessment, 0.1%
NaN$_3$ was added to the concentrated B-BSA to discourage
microbial growth. Hapten numbers in the range from 2 to 9
were obtained. Before spotting these solutions using the ink
jet system, an aliquot of a concentrated biotin-BSA solution
25   was diluted to 40 μg/mL in PBS and degassed.

In results to be discussed below, the biotin-BSA has
been further derivatized to include a benzophenone moiety that
may be activated with ultraviolet light to form a covalent
bond with the acrylic test surface. To prepare this Biotin-
30   BSA-benzophenone, 1.0 mL of biotin-BSA (5 mg/mL in PBS) was
combined with 68 μL of LC-SPDP (Pierce #21652, 10 mg/mL in
DMF) and allowed to react for two hours at room temperature,
followed by overnight dialysis vs. PBS. The following day, 40
μL of 100 mM dithiothretol was added to the B-BSA-SPDP and the
35   reaction proceeded for 1 hour at room temperature before being
purified on a PD 10 column (Pharmacia) in PBS. The B-BSA-SH
was collected in a 2.0 mL fraction. Titration for SH content

with Ellman's reagent showed an SH/BSA ratio of 8.0. To the
B-BSA-SH fraction (4.75 mg / 1.95 mL PBS), 50 μL (8.8 mg/mL in
DMF) of benzophenone-4-maleimide (Molecular Probes #B-1508,
Lot 8A-5) was added. This mixture reacted for two hours at

5   room temperature. Again titrating for SH content, it was
found that the residual SH/BSA content was 0.36, indicating
that benzophenone incorporation was near 7.5 per BSA. The
balance of the Biotin-BSA-Benzophenone material purified by
overnight dialysis vs. PBS.

10   D.   Application of Biotinylated BSA solutions to Acrylic
               Test Articles

               While the method of detection places constraints on
       the spot size and spotting format chosen, Fig. 3 (where
       protein adsorption is visualized via SA-HRP binding to

15   adsorbed biotin-BSA, followed by metal DAB precipitation –
       representative materials and methods below) shows that liquid
       volumes in the range of 200 pL to 200 nL, with corresponding
       spot sizes in the 0.05 (1 droplet) to 1.0 mm (1000 droplets)
       range, may easily be achieved in a desired pattern using this

20   embodiment of ink jet technology. Moreover, the use of
       multiple printing heads allows the formation of complex
       patterns of different materials which can be deposited
       simultaneously onto the test surface. For the interferometric
       detection scheme preferred in this invention, acrylic test

25   disks were typically spotted with 40 μg/mL B-BSA in PBS in 32

       or 64 circumferentially-spaced spots per revolution (at 0.4
       inches radius) with a drop-per-spot setting ensuring 50%
       coverage of surface along the circumference.

**E.   Washing Protein-coated Articles to Remove Excess
       Materials.**

Washing of excess buffer/protein materials
(typically with PBS/0.1% Tween 20) from ink jet spotted disks
5   is required so that only an adsorbed (or covalently-attached)
monolayer (or a few monolayers) of B-BSA is left on the
acrylic surface for subsequent chemistry steps. This washing
process removes most of the protein that was originally
spotted (ca 99%). To minimize spot smearing, one needs to
10   promote desorption off of the disk rather than across the
disk. Modifying the washing solution, and hence the solvency
of it, can be coupled with vigorous fluid – disk contact to
optimize this cleaning process. Allowing disks to dry before
washing also lessens smearing problems. Once spotted, dried,
15   and washed, the protein-coated disks were stable for (at
least) several weeks for use in immunoassay development.

**F.   Retention of Biotinylated BSA to Acrylic Test
       Articles**

       1. Liquid-phase adsorption as an example

20   Classically, protein adsorption is performed under
stagnant liquid-phase conditions, where binding between the
protein and the surface is achieved over a several hour time
scale (at room temperature) and the tightness of binding, or
resistance to desorption, increases with adsorption time
25   (Figure 4).

Materials:

       Biotinylated-BSA: see "C. Preparation of Biotinylated BSA
       solutions" above. This material was diluted to 40 μg/mL
       in PBS before use.

30   Assay Diluent: 0.1% BSA (Pentax), 0.05% Tween® 20
       (Mallinckrodt) in 10 mM PBS, 0.2 μm filtered (Gelman
       acrodisc).

       Wash Buffer: 0.1% Tween® 20 (Mallinckrodt) in 10 mM PBS.

SA-AP Conjugate: Streptavidin-alkaline phosphatase (SA-AP, Pierce, Product # 21324) was reconstituted to 1 mg/mL and frozen. Prior to use, the stock was further diluted in the 1:1000 to 1:1000000 range with Assay Diluent.

5   PNPP Kit: a tablet of PNPP (Sigma, N-2770) was reconstituted in the suggested buffer immediately prior to use.

Rings: Rings several millimeters thick and 3/8 in (I.D.) diameter were affixed to the clean, unspotted, 35 mm
10   acrylic test articles (Germanow-Simon) with double-stick tape to facilitate containment of liquid materials above the test surface.

Procedure:
1)   Clean disks with affixed rings had 500 μL of
15   biotinylated BSA added to each well.

2)   At various time afterward, the excess biotinylated BSA was removed and the wells were rinsed several times with Wash Buffer.

3)   Wells were then filled with 500 μL of Assay
20   Buffer for various times to promote desorption of previously adsorbed materials. Once the desorption phase was complete, the wells were rinsed with deionized water and allowed to dry.

4)   The SA-AP conjugate (at a 1:200 dilution to 5
25   μg/mL) was then added to the wells on the disks in a submilliliter volume (typically 250 μL) and gentle mixing was initiated.

5)   After a predetermined time (typically the 60 to 90 minutes required for the reaction to come to
30   completion), excess SA-AP was removed from the wells and

the wells were then washed with a back-and-forth motion in
two cups of wash buffer (several strokes in each cup).

6)   The metal PNPP substrate (diluted as
instructed) was then added to the wells (150 μL) and
allowed to react for 20 minutes without agitation.

7)   The reaction was stopped by adding 3 N NaOH (50
μL per well).

8)   A Molecular Devices microplate reader (E_{MAX})
was used to determine absorbance at 405 nm.

Results:

The results are set forth in Figure 4.

2. Adsorption after ink jetting, normal drying

The protein-binding process after ink jet-based
deposition of protein-based solutions onto the acrylic disk
actually consists of a least two phenomena - visual drying of
the spotting fluid and the associated process of adsorption
(or covalent linking, with associated UV-curing chemistry) of
a portion of the spotted B-BSA to the acrylic surface. Disk
drying time depends on spot size, fluid composition,
temperature, and humidity. For water-based vehicles spotted
at ambient conditions in the 32 spot format (ca. 0.2 μL per
spot), the crusting time is on the order of 10 to 30 minutes.
Adsorption, as indirectly measured by SA-HRP-DAB assay after
ink jet deposition (Figure 5) occurs on a longer (several
hour) time scale that is not unlike that for liquid phase
adsorption (Figure 4).

Materials:

Assay Diluent: 0.1% BSA (Pentax), 0.05% Tween® 20
(Mallinckrodt) in 10 mM PBS, 0.2 μm filtered (Gelman
acrodisc).

Wash buffer: 0.1% Tween® 20 (Mallinckrodt) in 10 mM PBS.

SA-HRP Conjugate: Streptavidin-horse radish peroxidase (SA-HRP, Pierce, Product No. 21127) was reconstituted to 1 mg/mL and frozen. Prior to use, the stock was further diluted in the 1:1000 to 1:1000000 range with Assay Diluent.

Metal Enhanced Diaminobenzidine (DAB) Kit: (Pierce, #34065) a 10X metallic solution was diluted in the provided hydrogen peroxide diluent about 1 hour prior to use.

Rings: One with a diameter similar to the outer diameter of the disk and about 2 mm thick, and the other with an approximately 0.5 inch diameter (and similar thickness) were affixed with double stick tape. This allowed containment of liquid between the rings in order to perform the assay.

Procedure:

1) Spotted, washed (after various amounts of post-spotting time), and dried disks had two rings affixed to them with double-stick tape and then were placed on a rotary mixer.

2) The SA-HRP conjugate (at a suitable dilution) was then added to the disks in a submilliliter volume (typically 0.5 mL) and gentle mixing was initiated.

3) After a predetermined time (typically the 60 to 90 minutes required for the reaction to come to completion), excess SA-HRP was removed from the disk and the disks were then gently washed with a back-and-forth motion in two cups of wash buffer (several strokes in each cup).

4) The metal DAB precipitating enzyme substrate was then added to the disk and allowed to react for a set time in the range of 10 to 30 minutes without agitation.

5)   The reaction was stopped by DI-water washing the
disks to remove the metal DAB solution. After washing,
some disks were dried with heat (typically, a hair
dryer).

5   6)   An interferometer was used to determine the amount
of material precipitated and attached to the disk, as
described in detail in copending application serial
number 08/298,998, the disclosure of which is
incorporated herein by reference.

10   **Results:**
The results are set forth in Figure 5.

3. Adsorption after ink jetting, heat acceleration
Heating (in the 70º to 90º C regime, with the upper
temperature limited by the softening of the acrylic surface)
15   after the spotting process accelerates the protein binding
process from hours to minutes. For example, heating freshly
spotted disks to 90º C for 30 minutes and then developing them
with the SA-HRP-DAB assay shows that essentially the same
interferometric signal is attained as for disks that had been
20   aged at room temperature for several days. In addition, heat
treating aged disks in a similar fashion has little, if any,
influence on the eventual interferometric signal (Figure 6).
In other words, hours of ambient drying/adsorption are
apparently functionally equivalent to minutes of heating.
25   This suggests that either a similar phenomena is occurring due
to the heating process (time - temperature superposition) or
different mechanisms (such as crosslinking, etc. for the
heated samples) are occurring but the surface area for protein
- plastic interactions governs maximal binding.
30   **Materials and Procedure:**
Same as "2. Adsorption after ink jetting, normal drying,"
above, except that the disks were heated in a small oven
in the 70º to 90º C range for 10 to 30 minutes after
spotting and before the disk washing process.

Results:

The results are set forth in Figure 6.

4. Protein binding after adsorption, UV-induced crosslinking

5   To accelerated the binding process and to maximize retention of proteins to the acrylic test surface, it is prudent to covalently link at least a portion of the deposited proteins to the test surface using bifunctional linking molecules (see "C. Preparation of Biotinylated BSA solutions"

10   above for details on preparing benzopohenone derivatized biotinylated-BSA). Table 1, below, presents data illustrating that about 10 minutes of UV illumination is sufficient to bind benzophenone-derivatized B-BSA to a level equivalent to overnight adsorption of B-BSA and that this covalently bound

15   material is highly resistant to detergent-induced desorption.

Materials:

Biotinylated-BSA: see "C. Preparation of Biotinylated BSA solutions" above. This material was diluted to 40 μg/mL in PBS before use.

20   Bennzophenone-derivatized, Biotinylated-BSA: see "C. Preparation of Biotinylated BSA solutions" above. This material was diluted to 40 μg/mL in PBS before use.

Assay Diluent: 0.1% BSA (Pentax), 0.05% Tween® 20 (Mallinckrodt) in 10 mM PBS, 0.2 μm filtered (Gelman

25   acrodisc).

Wash Buffer: 0.1% Tween® 20 (Mallinckrodt) in 10 mM PBS.

SA-HRP Conjugate: Streptavidin-horse radish peroxidase (SA-HRP, Pierce, Product #21127) was reconstituted to 1 mg/mL and frozen. Prior to use, the stock was further

30   diluted in the 1:1000 to 1:1000000 range with Assay Diluent.

o-Phenylenediamine Dihydrochloride (OPD) substrate: A
solution of 0.4 mg/mL OPD was made by dissolving a 15 mg
tablet of OPD (Sigma) into 37 mL of 0.1M citrate-
phosphate buffer, pH 5.0. Immediately prior to use, 10
mM hydrogen peroxide was added (37 μL of 30% solution,
Sigma) as an activating agent. Sulfuric acid (2N) was
added in an in an equal volume to OPD to stop the
reaction at the desired time.

Rings: Teflon Rings several millimeters thick and 0.25
inches in inner diameter were affixed to the clean,
unspotted, 35 mm acrylic test articles (Germanow-Simon)
with double-stick tape to facilitate containment of
liquid materials above the test surface. After the
protein adsorption step in the assay, the rings were
removed and a fresh ring and double-stick tape was
affixed to the original double-stick tape. This
eliminates assay activity due to "wall effects."

Procedure:

1)   Several clean disks with 2 affixed rings each
had 100 μL of biotinylated BSA added to one well and 100
μL of benzophenone-derivatized biotinylated BSA added to
the other.

2)   Half of the spotted disks were UV-illuminated (312
nm maximum, 5 Stratagene Stratalinker bulbs, product
#400187) for 10 minutes.

3)   2/3 of the disk lot was then washed several times
with Wash Buffer to remove excess biotinylated BSA and
then the wells were rinsed several times with deionized
water. Half of these disks were then stored while the
other half were contacted 12 hours with Wash Buffer in a
paddle washer (46 rpm) to stimulate desorption.

4)   The remaining 1/3 of the disk lot was stored
overnight with the biotinylated BSA solutions still in

26

contact with the disk surface. They were then washed
several times with Wash Buffer to remove excess
biotinylated BSA and then the wells were rinsed several
times with deionized water.

5   5)   Teflon rings were then replaced with new ones
and a new piece of double-stick tape.

           6)   The SA-HRP conjugate (at a 1:50000 dilution)
was then added to the wells on the all the disks in a 100
μL volume.

10   7)   After 60 minutes, excess SA-HRP was removed from the
wells and the wells were then washed with a back-and-
forth motion in two cups of wash buffer (several strokes
in each cup).

           8)   The freshly-prepared OPD substrate was then
15 added to the wells (50 μL) and allowed to react for 10
minutes without agitation.

           9)   The reaction was stopped by adding 50 μL of 2 N
H_2SO_4.

           10) A microplate reader was used to determine absorbance
20 at 490 nm.

Results:
           The results are set forth in Table 1.

TABLE 1

Acceleration of biotin-BSA binding, and resistance to detergent-induced desorption, by UV photoactivation. SA-HRP-OPD Assay - see text.

| | A-490 Signal | |
	Normal	UV Treated
5 minutes protein contact:		
B-BSA	0.434	0.617
B-BSA-Bph	0.619	0.720
5 minutes protein contact, then overnight desorption:		
B-BSA	0.245	0.349
B-BSA-Bph	0.281	0.761
Overnight (ca. 15 hours) protein contact:		
B-BSA	0.736	0.719
B-BSA-Bph	0.609	0.675

5.   CKMB Assay Performed using Invention

A preferred embodiment of this invention is to determine CKMB levels in suspected AMI patients. This method, assayed spectrophotometrically or interferometrically, is able to rapidly detect CKMB in clinically-significant levels (Figure 7).

Materials:

Assay Diluent: 0.1% Bovine Serum Albumin (BSA) (Pentax), 0.05% Tween® 20 (Mallinckrodt) in 10 mM PBS, pH 7.4, 0.2 μm filtered (Gelman acrodisc).

Blocking Buffer: 0.3% BSA (Pentax), 0.05% Tween® 20 (Mallinckrodt) in 10 mM PBS, pH 7.4; 0.2 μm filtered (gelman acrodisc).

Wash Buffer: 0.05% Tween® 20 (Mallinckrodt) in 10 mM PBS, pH 7.4.

Ink-jet Spotted Disk with Rings: 32 spot disks that have been printed, washed and had rings affixed for fluid containment. See "2. Adsorption after ink jetting, normal drying."

Streptavidin-antiCKMB Conjugate: Streptavidin/antiCKMB
conjugate was prepared using S-acetylthioglycolic acid N-
hydroxysuccinimide ester (SATA, Pierce #26102), a
heterobifunctional linking reagent from Sigma and
5   Succinimidyl 4-(N-maleimidomethyl)
cyclohexane-1-carboxylate (SMCC, Pierce #22320) from
Pierce. Fifteen (15) molar excess of SATA (dissolved at
5 mg/mL in dimethylformamide (DMF) from Pierce) was
reacted with 1 mg of antiCKMB at 0.74 mg/mL in PBS at
10   pH 7.4 for 3 hours at room temperature. Also, 15 molar
excess of SMCC (dissolved at 5 mg/mL in DMF) was reacted
with 1.1 mg of streptavidin at 1.1 mg/mL in PBS for 3
hours at room temperature. Unreacted linkers were
removed from both antiCKMB/SATA and streptavidin/SMCC
15   reaction mixtures using Sephadex® G25M column (PD10
column from (Pharmacia) and eluting with 3 mL of 10 mM
PBS at pH 7.4. The purified antiCKMB-SATA and
streptavidin-SMCC were mixed at the protein molar ratio
of 1:3, resulting in the total reaction volume of 6mL.
20   The conjugation reactin was initiated by adding 1 M
hydroxylamine to a final concentration of 100 mM and
incubated over 18 hours at 4°C. The reaction was stopped
by adding 100 mM N-ethylmaleimide (NEM) from Aldrich
Chemical at 1 mM final concentration in the reaction
25   mixture and incubating for 15 minutes at room
temperature. After the incubation with NEM, the
conjugation reaction mixture was concentrated 3-fold, and
the final volume was reduced to 2 mL using a Centricon-
100 concentrator from Amicon. Then the concentrated
30   mixture was purified using a Bio-Gel® A-5m column from
Bio-Rad to isolate streptavidin-antiCKMB conjugate.

Anti-CKMM-Horse Radish Peroxidase (HRP) Conjugate:
AntiCKMM-HRP conjugate was made in two steps as follows.
First, goat antiCKMM antibody (BiosPacific #G31520) was
35   conjugated to SATA (Sigma) at a molar ratio of SATA to
antibody of 20:1. 4 mg of antibody required 0.1156 mg of
SATA, which was obtained by adding 5.2 mg of SATA to

500 μL of DMF (Pierce) for a 10.4 mg/mL solution.   The
4 mg of goat antiCKMM was diluted to 2.5 mM with PBS,
pH 7.3, and 11 μL of SATA was added and incubated at room
temperature for 3 hours. HRP (Sigma) was conjugated to
SMCC (Pierce) at a molar ratio of SMCC to HRP of 20:1.
10 mg of HRP was diluted to 2.5 mL in PBS, pH 7.3 10 mg
of HRP required 1.67 mg of SMCC. 65 mg of SMCC was
dissolved in 100 μL of DMF for a 65 mg/mL solution.
26 μL of the SMCC solution was added to the HRP in PBS
and incubated for 3 hours.

        Before the second step, the HRP/SMCC and goat
antiCKMM/SATA were each placed on a PD-10 column
(Pharmacia) which had been equilibrated in PBS, pH 7.3 to
remove the free SATA and SMCC. The goat antiCKMM was
eluted with 3 mL of PBS while the HRP was eluted with
2.8 mL of PBS. This 5.8 ml volume was placed together
and 580 μL of 1M hydroxylamine, pH 7.0 (Sigma) was added
to start the conjugation which continued at 4°C
overnight. The reaction was then stopped by adding 64 μL
of a 12.5 mg/mL solution of NEM (Aldrich) in DMF and
incubating for 15 min. at room temperature. The 6+ mL
were then concentrated in a Centricon 30 concentrator to
2 mL and fractionated over a 100 cm Bio-Gel® A-5M column.

CKMB Calibrators: CKMB (Fortron, #I-028) was diluted to
prepare 0, 10, 100 ng/mL calibration solutions.

HRP Substrates:
A)   o-Phenylenediamine Dihydrochloride (OPD), 0.4 mg/mL
made by dissolving 15 mg tablet of OPD (Sigma) into 37 mL
of 0.1M citrate-phosphate buffer, pH 5.0. Just prior to
addition onto the disk, 10 mM hydrogen peroxide was added
(37 μL of 30% solution, Sigma). Sulfuric acid (2N) was
used to stop the reaction at the desired time.

30

B)   *Metal Enhanced Diaminobenzidine* (DAB) *kit* (Pierce,
#34065). A concentrated 10X metallic solution was
diluted in hydrogen peroxide diluent (0.15 mL of
concentrate was added to 1.35 mL of diluent).

5   **Procedure:**

1)   200 μL of blocking buffer was added to the annulus
of each disk. All disks were then placed in 37°C
incubator for 10 minutes and mixed with rotomixer.

10

2)   All disks were then washed in Wash Buffer with
a back-and-forth motion in several cups.

3)   200 μL of diluted streptavidin-antiCKMB was
added into each disk. All disks were placed back
onto the rotomixer in the incubator for another
10 minutes.

15

4)   All disks were again washed in Wash Buffer.

5)   200 μL of the CKMB calibrators were added into
the disks. All disks were again incubated for 10
minutes as described above.

6)   All disks were again washed in wash buffer.

20

7)   200 μL of diluted antiCKMM-HRP was measured
into each disk. All disks were again incubated for
10 minutes as in previous manner.

8)   All disks were again washed in wash buffer.

9)   Enzyme substrate was added at room temperature.

25
         A)   100 μL of the hydrogen peroxide
activated OPD was added onto each of the
disks in the first set.

B)   200 µl of the diluted Metal DABN
        solution was added onto each of the discs
        in a second set.

10) Following a 10 minutes incubation, the OPD
disks were stopped with the addition of 100 µl of
the 2N sulfuric acid and the DAB disks were washed
off/immersed in three cups of distilled water.

11) The OPD coloring solution (200 µL) was
transferred to a microplate and read with microplate
reader (Molecular Devices, E_{MAX}) at 490 nm. The DAB
disks were dried and measured with the
interferometer.

Results:

The results are set forth in Figure 7.

Although the foregoing invention has been described
in some detail by way of illustration and example, for
purposes of clarity of understanding, it will be obvious that
certain changes and modifications may be practiced within the
scope of the appended claims.

WHAT IS CLAIMED IS:

1.   A method for immobilizing a hapten on a solid phase surface, said method comprising applying to the surface a liquid medium including the hapten attached to a carrier molecule under conditions so that the carrier molecule adheres to the surface and the hapten remains active and sterically available for binding to a specific binding partner.

2.   A method as in claim 1, wherein the hapten is a small molecule having a molecular weight below 2000 Daltons and the carrier molecule is a macromolecule having a molecular weight above 10 kD.

3.   A method as in claim 2, wherein the hapten is selected from the group consisting of biotin, fluorescein, dinitrophenol, digoxin, luminol, theophylline, and morphine.

4.   A method as in claim 2, wherein the macromolecule is a protein selected from the group consisting of albumins, immunoglobulins, thyroglobulins, and ferritin.

5.   A method as in claim 1, wherein the hapten is covalently attached to the carrier molecule.

6.   A method as in claim 5, wherein the hapten is covalently attached through at least one linking moiety.

7.   A method as in claim 1, further comprising cross-linking the carrier molecule to the solid phase surface after the carrier molecule has initially adhered by non-covalent interaction.

8.   A method as in claim 1, wherein the liquid medium is applied as a spray of discrete droplets each having a volume in the range from 10 pl to 1 μl.

1   9.   A method as in claim 1, wherein the liquid
2   medium is applied by pipetting a volume of the liquid medium
3   in the range from 200 pl to 100 μl.

1   10.   A method as in claim 1, wherein the hapten is
2   immobilized over a discrete reaction zone on the solid phase
3   surface, having an area in the range from 0.1 mm^2 to 100 mm^2.

1   11.   A method as in claim 1, wherein a plurality of
2   discrete reaction zones are formed in a preselected pattern on
3   the solid phase surface.

1   12.   A method as in claim 1, wherein the solid phase
2   surface is composed of a material selected from the group
3   consisting of organic polymers, glasses, ceramics, metals, and
4   paper materials.

1   13.   A method as in claim 1, further comprising
2   binding a specific binding substance to the hapten on the
3   solid phase.

1   14.   A method as in claim 13, wherein the specific
2   binding substance is attached to a hapten-binding substance
3   and wherein binding occurs through the hapten and the hapten-
4   binding substance.

1   15.   A method for immobilizing a hapten on a solid
2   phase surface, said method comprising:
3   providing a hapten attached to a carrier molecule
4   present in a liquid medium; and
5   applying a preselected volume of the liquid medium
6   against the solid phase surface so that the carrier molecule
7   non-covalently adheres to said surface to form a reaction
8   zone; and
9   cross-linking the adhered carrier molecule to the
10   solid phase surface.

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1   16.   A method as in claim 15, wherein said cross-
2   linking step comprises exposing the solid phase surface to
3   radiation which effects cross-linking between a moiety
4   attached to the carrier molecule and the surface.

1   17.   A method as in claim 15, wherein the volume of
2   liquid medium is applied by spraying a plurality of droplets
3   having individual volumes in the range from 10 pl to 1 μl
4   propelled by maintaining the liquid medium in a chamber and
5   inducing pressure pulses to force the droplets through an
6   aperture toward the solid phase.

1   18.   A method as in claim 15, wherein the volume of
2   liquid medium is applied by pipetting a volume in the range
3   from 200 pl to 100 μl.

1   19.   A method as in claim 15, further comprising
2   heating the solid phase surface to a temperature and for a
3   time selected to enhance adherence of the carrier molecule to
4   the surface.

1   20.   A method as in claim 15, wherein the aperture
2   is moved relative to the solid phase surface in order to
3   immobilize hapten over a discrete reaction zone on the solid
4   phase surface, having an area in the range from 0.1 mm^2 to 5
5   mm^2.

1   21.   A method as in claim 20, wherein from 1 droplet
2   to 10,000 droplets are propelled against the solid phase
3   surface to produce each reaction zone.

1   22.   A method as in claim 17, wherein the aperture
2   is moved relative to the solid phase surface in order to form
3   a plurality of discrete reaction zones in a preselected
4   pattern on the solid phase surface.

1   23. A method as in claim 15, wherein the hapten is
2 a small molecule having a molecule weight below 2000 Daltons
3 and the carrier molecule is a macromolecule having a molecular
4 weight above 10 kD.

1   24. A method as in claim 23, wherein the hapten is
2 selected from the group consisting of biotin, fluorescein,
3 dinitrophenol, digoxin, luminol, theophylline, and morphine.

1   25. A method as in claim 23, wherein the
2 macromolecule is a protein selected from the group consisting
3 of serum albumins, immunoglobulins, thyroglobulins, and
4 ferritin.

1   26. A method as in claim 15, wherein the hapten is
2 covalently attached to the carrier molecule.

1   27. A method as in claim 16, wherein the moiety is
2 selected from the group consisting of benzophenome and
3 fluorinated aryl azide, and wherein the exposing step
4 comprises exposing the surface to ultraviolet radiation.

1   28. A method as in claim 15, wherein the solid
2 phase surface is composed of a material selected from the
3 group consisting of organic polymers, glasses, ceramics,
4 metals, and paper materials.

1   29. A method as in claim 15, further comprising
2 binding a specific binding substance to the hapten on the
3 solid phase.

1   30. A method as in claim 28, wherein the specific
2 binding substance is attached to a hapten-binding substance
3 and wherein binding occurs through the hapten and the hapten-
4 binding substance.

1   31. A test article comprising:

2   a solid phase having a test surface;

3   hapten attached to a carrier molecule immobilized to

4   the test surface wherein the hapten is linked to the surface

5   by the carrier molecule.

1   32. A test article as in claim 31, wherein the

2   solid phase surface is composed of a material selected from

3   the group consisting of organic polymers, glasses, ceramics,

4   metals, and paper materials.

1   33. A test article as in claim 31, wherein the

2   hapten is a small molecule having a molecular weight below

3   2000 Daltons and the carrier molecule is a macromolecule

4   having a molecular weight above 10 kD.

1   34. A test article as in claim 33, wherein the

2   hapten is selected from the group consisting of biotin,

3   fluorescein, dinitrophenol, digoxin, luminol, theophylline,

4   and morphine.

1   35. A test article as in claim 33, wherein the

2   macromolecule is a protein selected from the group consisting

3   of serum albumins, immunoglobulins, thyroglobulins, and

4   ferritin.

5   36. A test article as in claim 31, wherein the

6   hapten is covalently attached to the carrier molecule.

1   37. A test article as in claim 36, wherein the

2   hapten is biotin covalently attached to a serum albumin

3   carrier molecule.

1   38. A test article as in claim 31, wherein the

2   carrier molecule is non-covalently attached to the test

3   surface.

1   39.   A test article as in claim 31, wherein the
2   carrier molecule is covalently cross-linked to the solid phase
3   surface.

1   40.   A test article as in claim 31, further
2   comprising a specific binding substance attached to the hapten
3   by a hapten-binding substance, wherein the specific binding
4   substance and hapten-binding substance are covalently attached
5   to each other.

1   41.   A test article as in claim 31, wherein the
2   hapten is biotin, the carrier molecule is a serum albumin, the
3   hapten-binding substance is avidin, and the specific binding
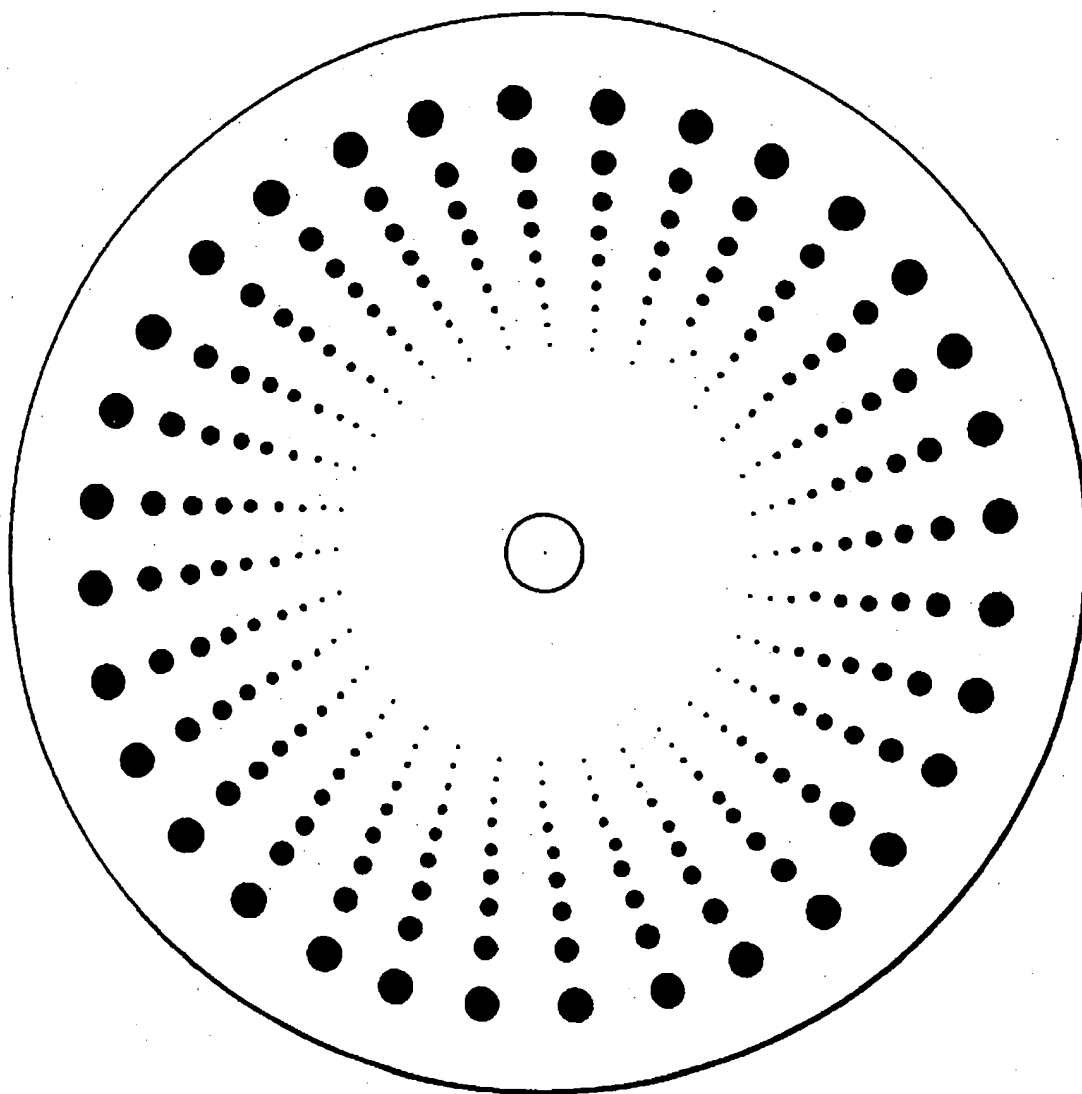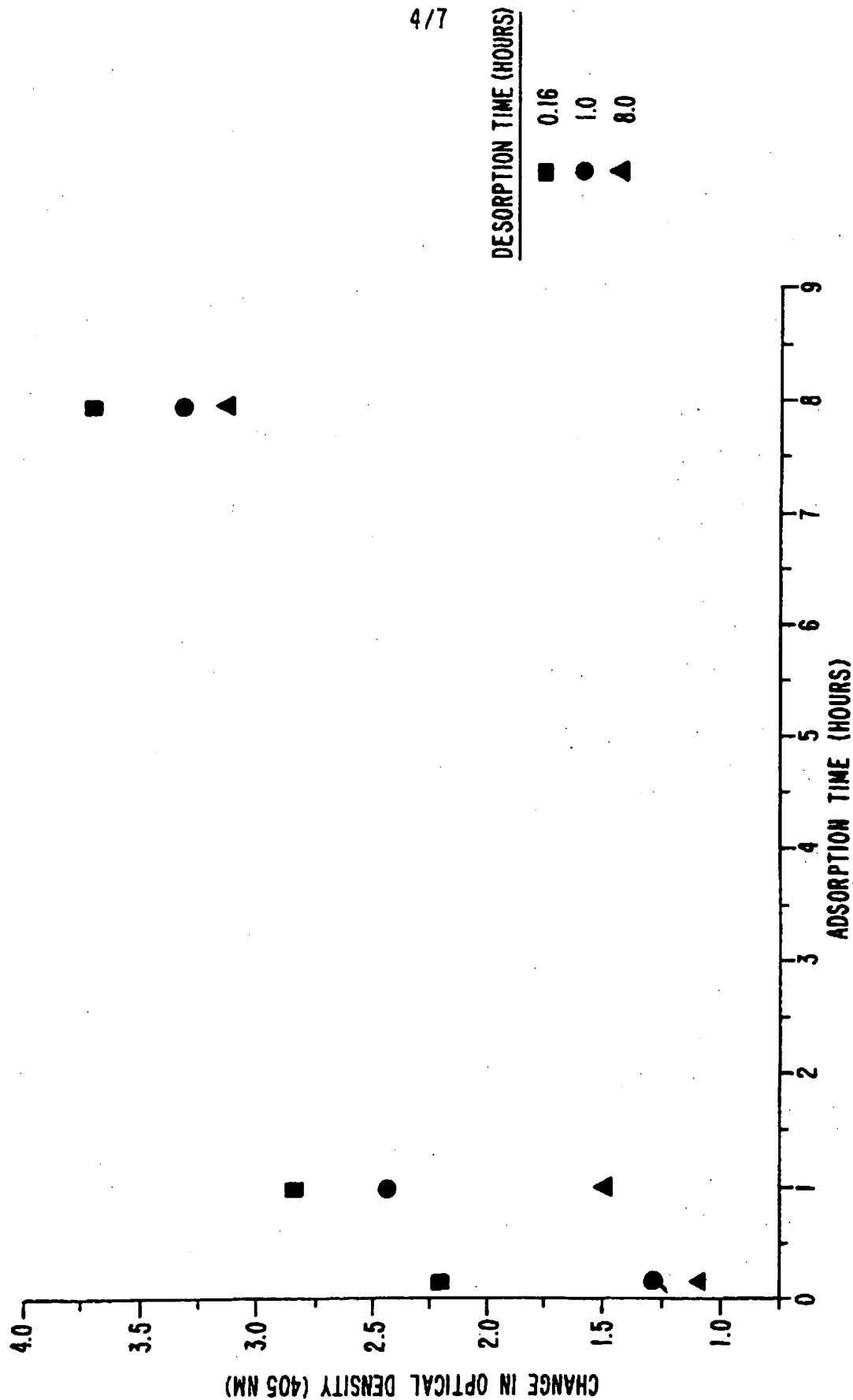4   substance is an antibody or antibody fragment.
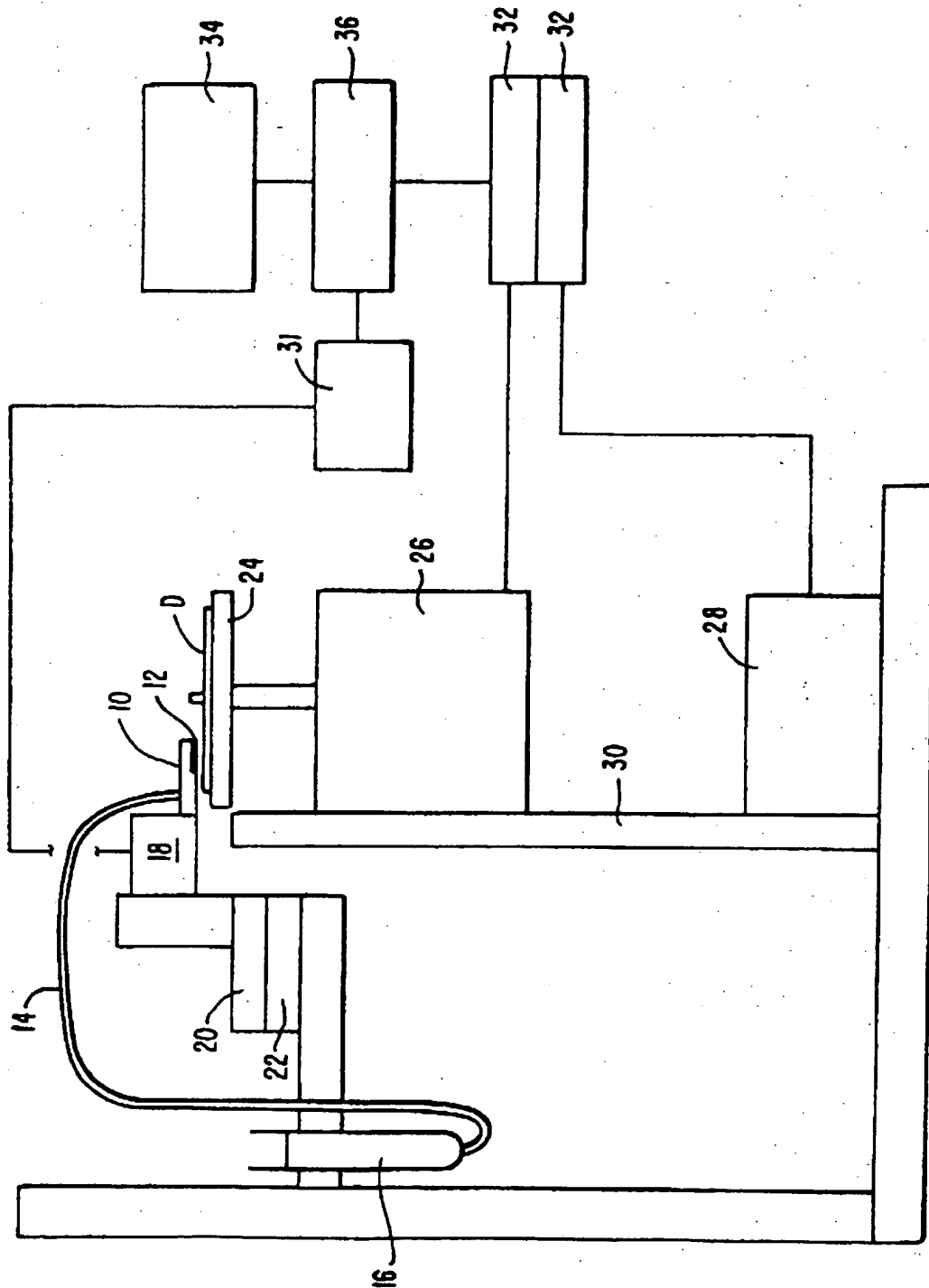
FIG. 1.
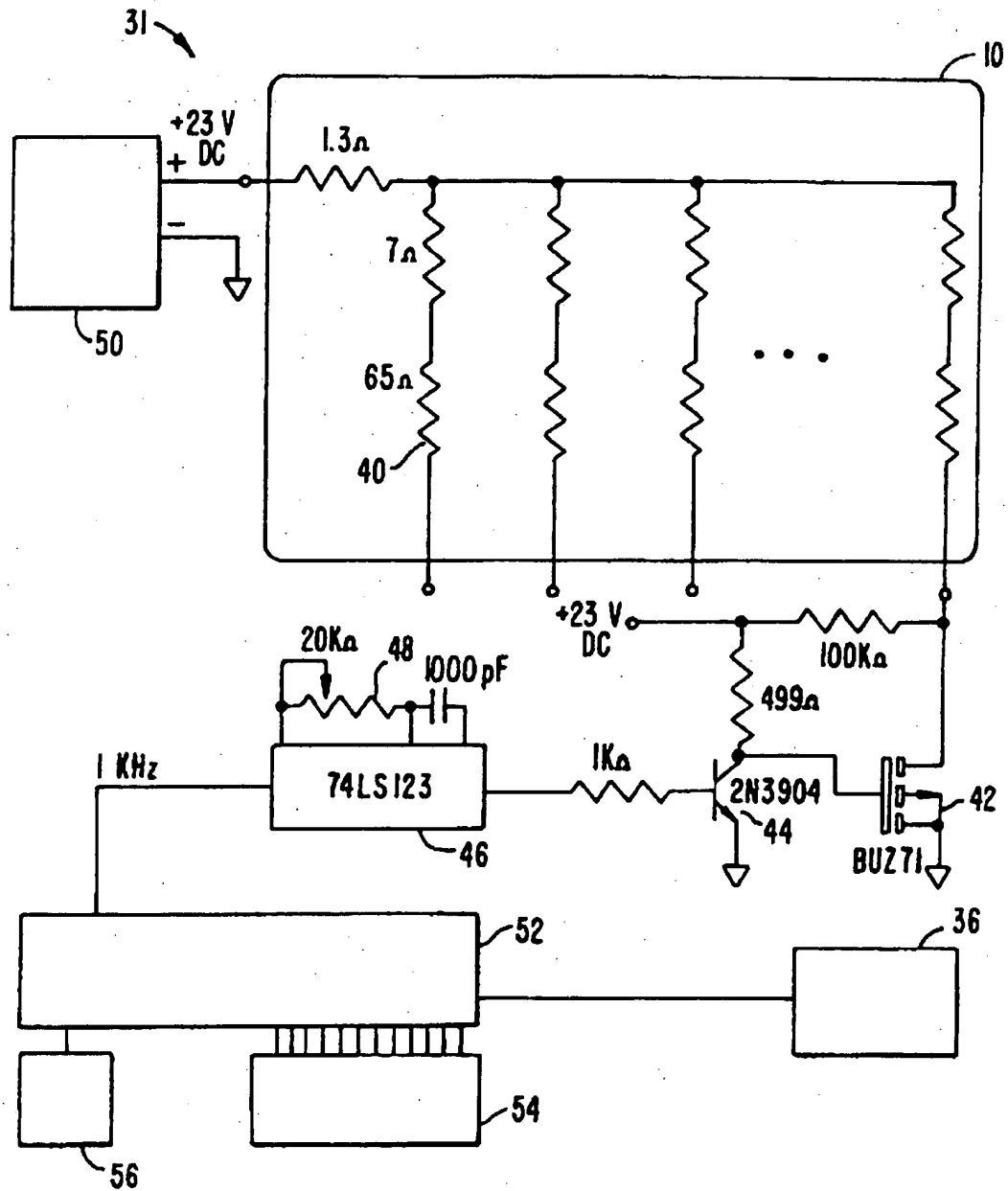
FIG. 2.

FIG. 3.
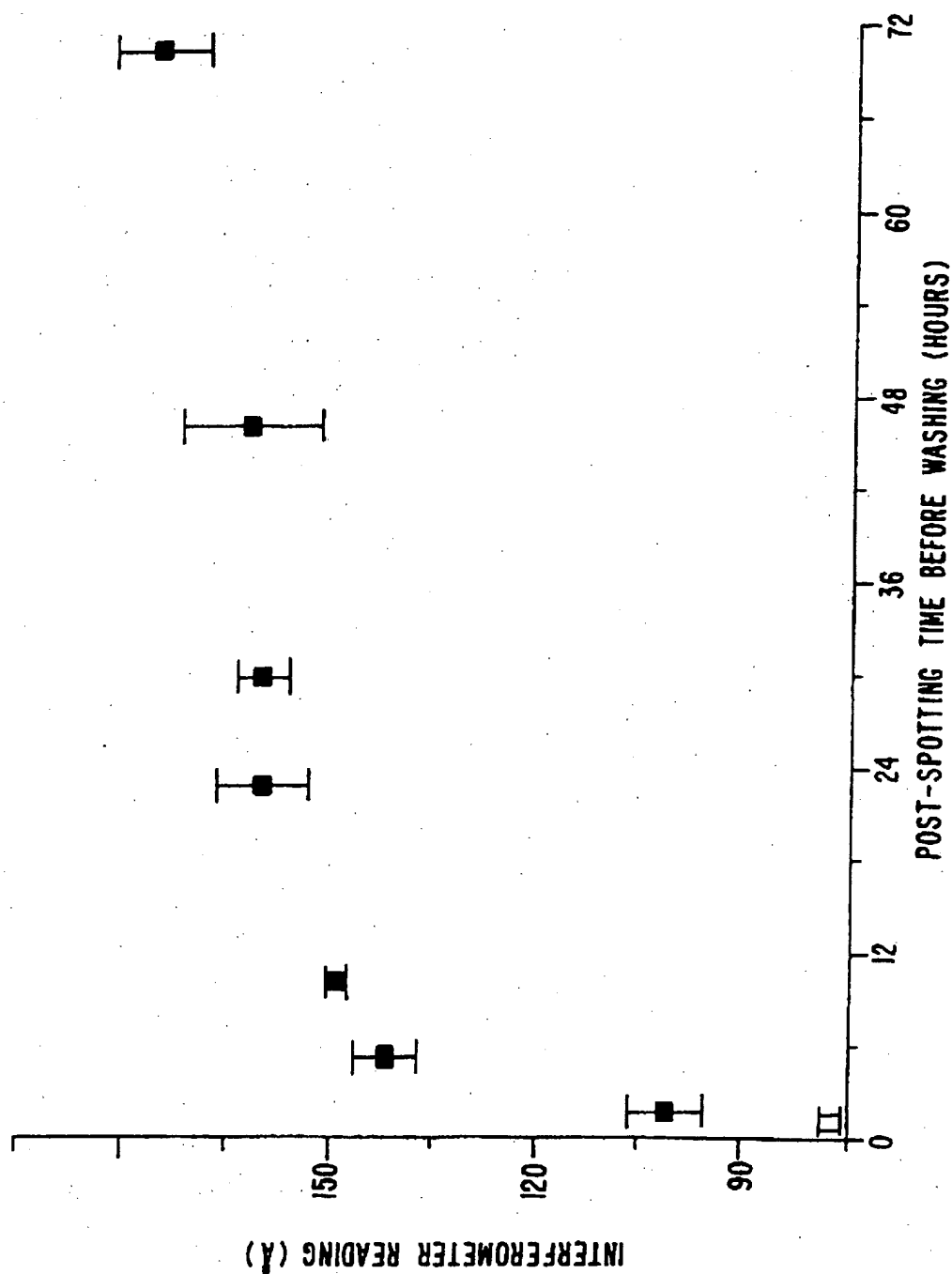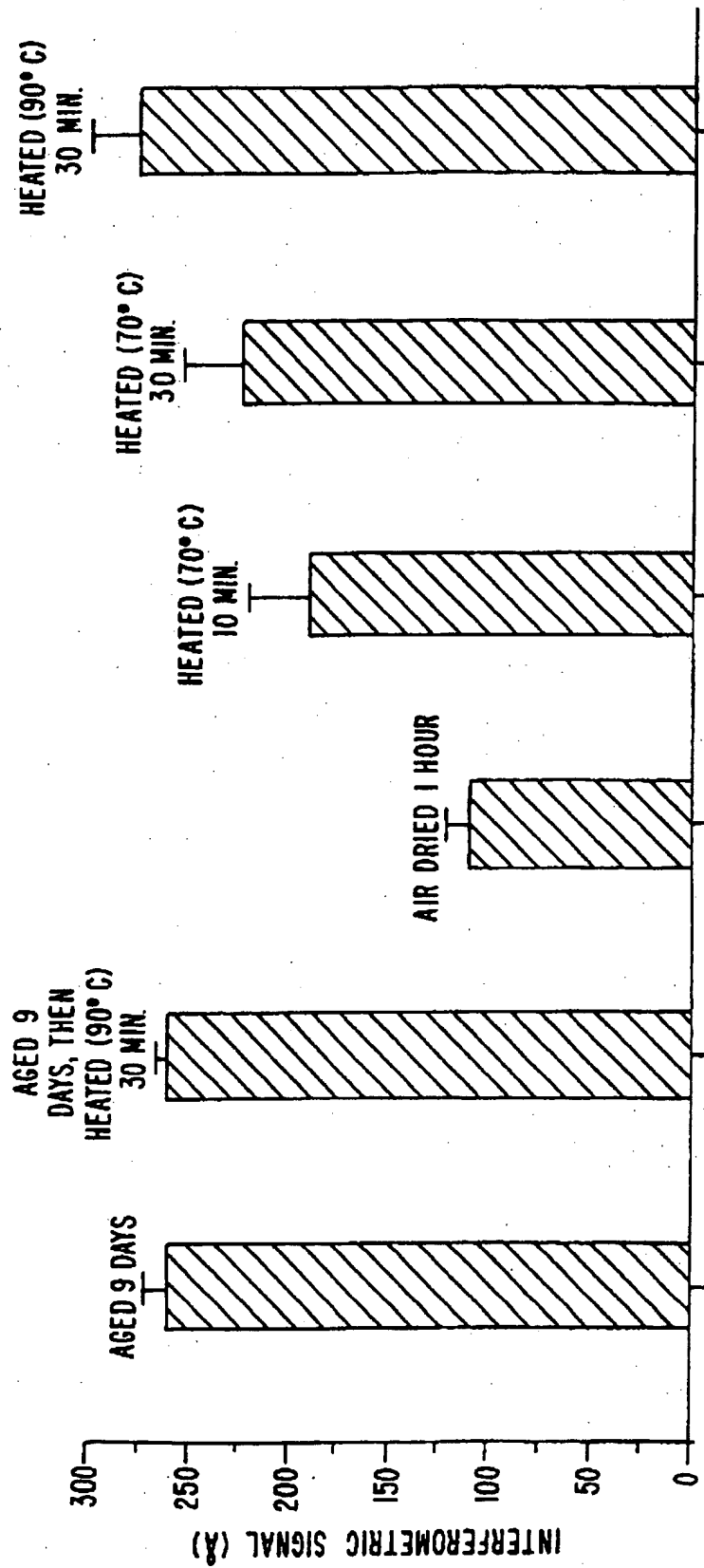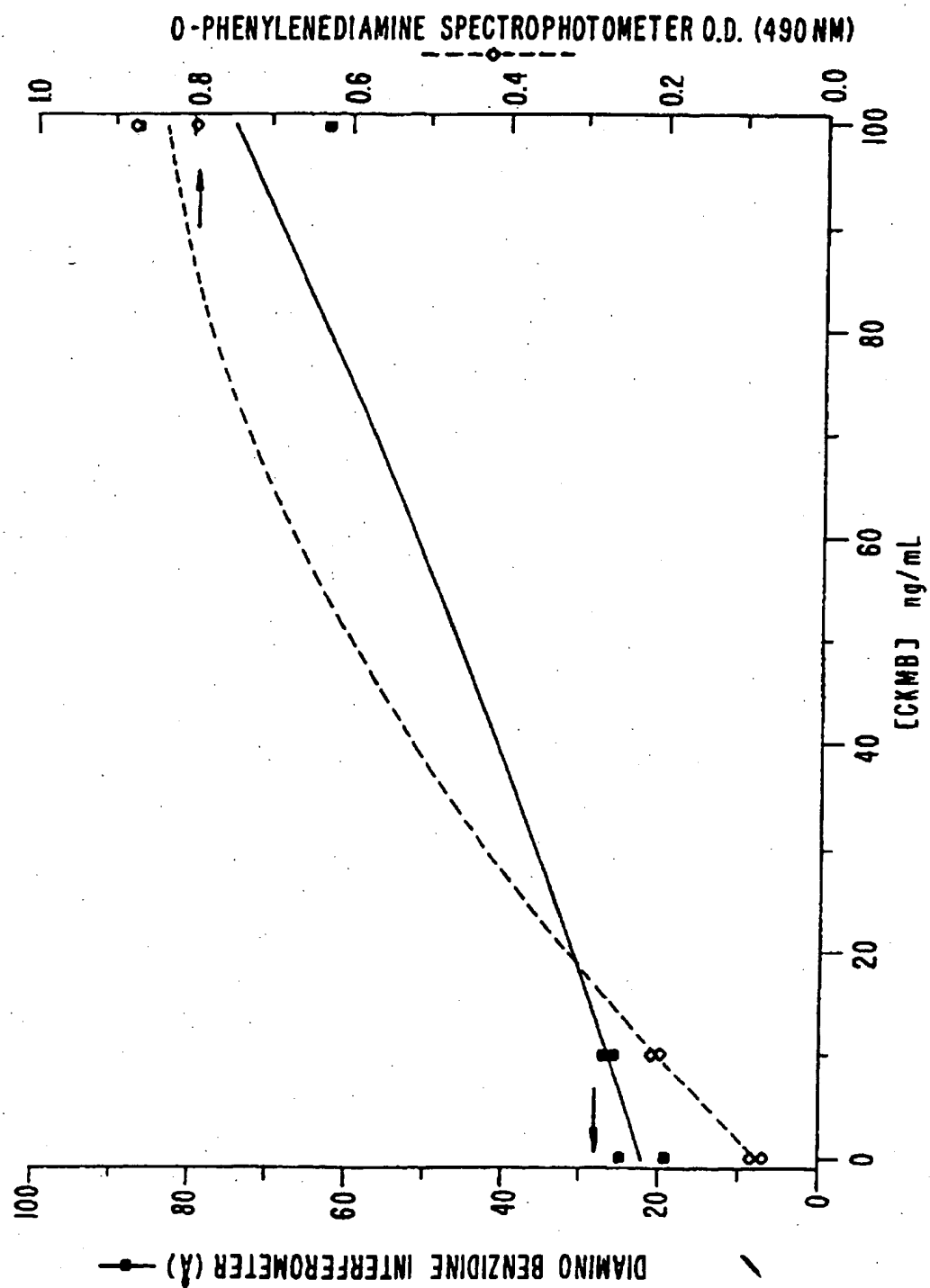
FIG. 4.

FIG. 5.

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FIG. 6.

FIG. 7.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/543
US CL :436/518, 524, 527, 528, 530, 532, 543; 422/57; 435/7.1, 805, 970

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/518, 524, 527, 528, 530, 532, 543; 422/57; 435/7.1, 805, 970

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,362,655 A (SCHENK ET AL) 08 NOVEMBER 1994, column 1, lines 52-61.	1-41
Y	US 4,791,067 A (SHEIMAN ET AL) 13 DECEMBER 1988, column 3, line 6- column 4, line 28.	1-41
X --- Y	US 5,045,480 A (JOHNSON ET AL) 03 SEPTEMBER 1991, columns 9-12.	1-6, 12-14, 31-37, 39-41 ---------- 7-11, 15-30, 38
Y	US 5,316,784 A (MAURER ET AL) 31 MAY 1994, columns 2-3.	1-41
Y	US 5,063,081 A (COZZETTE ET AL) 05 NOVEMBER 1991, columns 31-32, and columns 50-51.	1-41

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 APRIL 1996	**10 MAY 1996**

Name and mailing address of the ISA/US	Authorized officer
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4,960,692 A (LENTRICHIA ET AL) 02 OCTOBER 1990, column 3, lines 23-38.	1-41
A	US 5,279,955 A (PEGG ET AL) 18 JANUARY 1994, column 3, lines 19-29.	1-41
X,P --- Y,P	US 5,468,649 A (SHAH ET AL) 21 NOVEMBER 1995, see abstract.	1-2, 4, 5, 12, 31-33, 35-36 ---------- 8-11, 15-23, 25-26, 28
X --- Y	US 4,096,138 A (SCHERR) 20 JUNE 1978, column 4, lines 21-37, and column 9, lines 50-68 .	1-5, 13-14, 31, 33-36, 40 ---------- 7-12, 15-26, 28-30, 32, 37, 39, 41

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